Med. Sci. Res., 1992; 20, 863–865

Nicotine phase 2 metabolites in human urine – structure of metabolically formed *trans*-3′-hydroxycotinine glucuronide

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Keywords: Urine, nicotine, cotinine, *trans*-3′-hydroxycotinine, glucuronic acid conjugates, sulphuric acid conjugates, TSP-MS, NMR.

Introduction: Evidence for phase 2 metabolites as major urinary nicotine metabolites in humans has recently been presented by several authors [*e.g.*, 1–4]: conjugates of nicotine, cotinine, and 3′-hydroxycotinine were quantified in urine samples indirectly by measuring the increase in the amounts of nicotine, cotinine, and 3′-hydroxycotinine after enzymatic hydrolysis using β-glucuronidase. This led to the suggestion that nicotine phase 2 metabolites are glucuronides of nicotine and its respective phase 1 metabolites.

Caldwell *et al.* [5] recently carried out thermospray liquid chromatography/mass spectrometry (MS) of a smoker's urine and electrospray MS of a high performance liquid chromatography (HPLC) fraction of it. The retention time and mass spectra were compared with those obtained for the fully synthesised and spectrometrically characterised reference substance. They claimed that N-β-D-glucopyranuronosyl-(S)-(–)-cotininium inner salt is a major urinary nicotine phase 2 metabolite.

In a very recent study [6], we analysed a smoker's urine by HPLC after derivatisation with 1,3-diethyl-2-thiobarbituric acid (DETBA), which is selective for compounds containing unsubstituted pyridine nitrogen: in addition to large peaks derived from *trans*-3′-hydroxycotinine (THOC), nicotine (NIC), and cotinine (COT), a large peak representing nicotine phase 2 metabolites was found. This peak was not detectable in the DETBA assay when the urine was treated with an enzyme preparation containing β-glucuronidase and sulphatase. The products of the enzyme cleavage were mainly THOC (67%), COT (31%) and a small amount of NIC (2%).

The objective of the present study was to determine which substituents are conjugated to the nicotine phase 1 metabolites and at which position.

Materials and methods: CH_2Cl_2 was from Merck (Darmstadt, Germany), DETBA from Aldrich (Steinheim, Germany), S-nicotine, chloramine-T, chloramphenicol β-D-glucuronide, β-glucuronidase (*Helix pomatia* type HP-2) and arylsulphatase (*Helix pomatia*, type H-2) from the Sigma Chemical Co. (Deisenhofen, Germany), COT from Roth (Karlsruhe, Germany), and THOC from Dr G. Neurath (Institut für Biopharmazeutische Mikroanalytik, Hamburg, Germany).

The pooled human urine sample was randomly collected (male Caucasian smoker, approximate daily consumption 50 cigarettes, nicotine delivery 1 mg per cigarette). In order to remove most of the nicotine phase 1 metabolites, aliquots of the urine were alkalised by NH_4OH to *p*H 10.8 and extensively washed with CH_2Cl_2 (4 times, twice the sample volume). Hydrolysis of the CH_2Cl_2-washed urine sample was performed enzymatically and by acid and alkaline treatment. The conditions are given in Table 1. The nicotine metabolites before and after hydrolysis were analysed using the DETBA HPLC assay [6].

Partial purification of nicotine phase 2 metabolites for MS and NMR analysis was performed starting with 500 mL of the CH_2Cl_2-washed urine. The purification procedures consisted of size-exclusion chromatography on Sephadex G10 (Pharmacia, Freiburg, Germany), collection of the fractions representing the first DETBA-positive peak, chromatography of these fractions by cation-exchange HPLC on a Partisphere SCX cartridge (Whatman, Maidstone, UK), repetitive chromatography of the DETBA-positive fractions by ion-pair HPLC on Radial Pak C18 cartridge connected in series with a Nova-Pak C18 column (Waters, Eschborn, Germany), and repetitive chromatography of the DETBA-positive fractions by reverse phase HPLC on the above-mentioned C18 columns. Two DETBA-positive fractions were obtained. For NMR analysis, fraction 2, representing the major DETBA-positive peak, was further purified by hydrophilic interaction liquid chromatography on a polyhydroxyethyl-aspartamide column (ICT, Frankfurt, Germany).

Thermospray mass spectrometry (TSP-MS) was performed on a mass spectrometer coupled to a TSP interface (QUATTRO, Fisons Instruments, Mainz, Germany). The solvent was a mixture of methanol and 0.1 mol L^{-1} ammonium acetate in water (50/50, v/v). The sample was directly applied by loop injection.

NMR spectrometry was performed on a Bruker AC-P 300

Table 1: Hydrolysis of CH_2Cl_2-washed smoker's urine.

Treatment	Hydrolysis condition				Metabolite detected[a]			
	Activity (units mL^{-1})	*p*H	Incubation time (h)	Temperature (°C)	C	NIC	COT	THOC
β-glucuronidase	6,000	4.7	18	37	0	+	–	+
Arylsulphatase	1,000	7.0	18	37	0	+	+	+
Alkali	–	14.0	16	70	+ (↓)	0	+	0
Acid	–	1.0	16	70	+ (↓)	+	0	+

NMR spectrometer. ^{1}H-NMR- and H, H-COSY-90 spectra of the samples (approx. 250 µg in 0.5 mL CD_3OD) were recorded.

Results and discussion: In the washed urine, the peak representing nicotine phase 2 metabolites, designated C for conjugates in Table 1, completely disappeared after treatment with β-glucuronidase and with sulphatase but only partially decreased after acidic and alkaline hydrolysis (Table 1). The differences found between alkaline and acidic hydrolysis might indicate that COT is conjugated by different substituents and/or at different sites than THOC and NIC.

In the TSP-MS of the partially purified fraction 1 (Figure 1a), $[M + H]^+$ ions corresponding to sulphuric acid conjugates of COT (m/z 257) and THOC (m/z 273) were found. The assumption that these ions represent nicotine phase 2 metabolites is supported by the occurrence of fragments with m/z 177 (COT) and m/z 193 (THOC). Several other ions in this spectrum might represent amino acids still present in this fraction (*e.g.*, m/z 134: asparagine, m/z 150: methionine).

In the TSP-MS of fraction 2 (Figure 1b), $[M + H]^+$ ions corresponding to a glucuronide of THOC (m/z 369) and of its aglycon THOC (m/z 193) were found. The fragment at m/z 351 can be explained by the loss of water (18 Daltons).

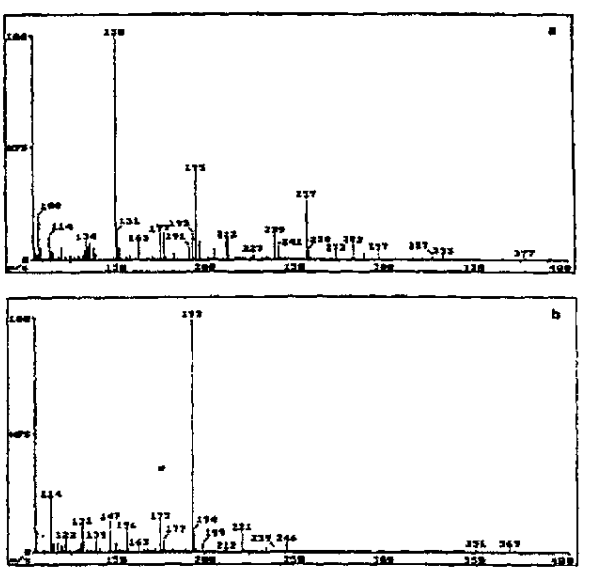


Figure 1: TPM-MS of partially purified nicotine phase 2 metabolites. a: fraction 1. b: fraction 2.

Table 2: Chemical shifts of pyridine, pyrrolidine and glucuronic acid ring protons.

H-No.	Chemical shift (ppm) THOC	Chloramphenicol-β-D-glucuronide	Fraction 2
2	8.4	–	8.4
4	7.7	–	7.7
5	7.5	–	7.5
6	8.5	–	8.5
3'	4.5	–	4.8
4'a,b	2.4, 2.3	–	2.6, 2.4
5'	4.8	–	4.8
6'	2.7	–	2.7
1"	–	4.4	4.4
2"	–	3.3	3.3
3"	–	3.4	3.4

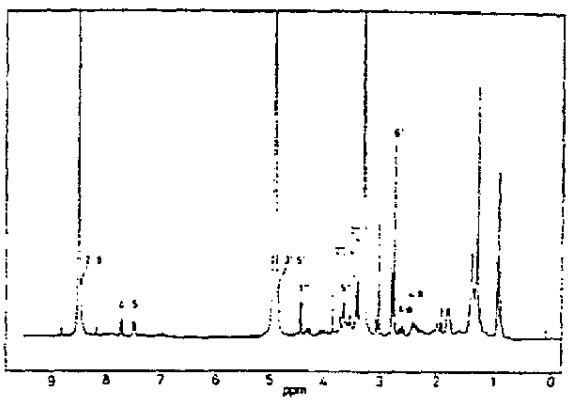


Figure 2: One-dimensional ^{1}H-NMR spectrum of partially purified nicotine phase 2 metabolites, fraction 2.

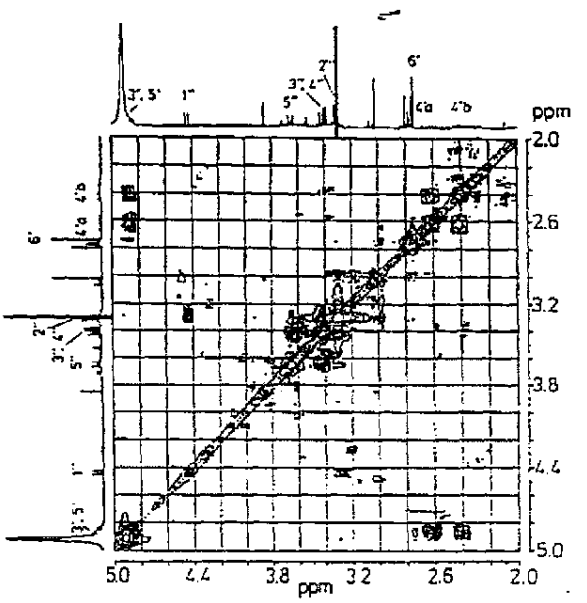
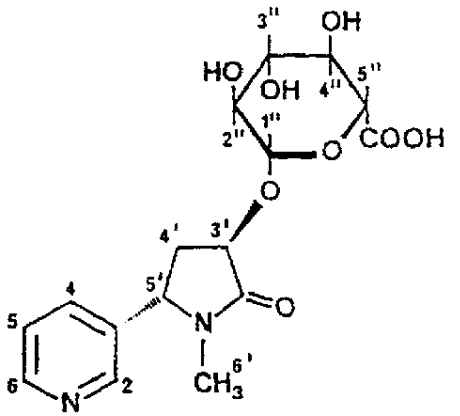


Figure 3: Two-dimensional H, H-COSY 90 spectrum of partially purified nicotine phase 2 metabolites, fraction 2.

In the one-dimensional ^{1}H-NMR spectrum (Figure 2) and in the H,H,-COSY 90 spectrum (Figure 3) of fraction 2, the signals of a substituted THOC were observed. A comparison of the chemical shifts with those of THOC (Table 2) shows that the substitution has to be at the oxygen of the hydroxyl-group at the 3'-position in the pyrrolidine ring. The ^{1}H-NMR-signals of the sugar moiety show complex unresolved multiplets.

Therefore, a straightforward analysis of this part of the spectrum was not possible. The structure of the sugar moiety, however, could be established by comparison with the spectrum of chloramphenicol-β-D-glucuronide, which showed similar chemical shifts for the glucuronic acid ring protons (Table 2). Spin simulation analysis based on the coupling constants obtained from the first order evaluation of this spectrum predicted fine structures for the multiplets of

in our urine sample the major nicotine phase 2 metabolite was *trans*-3'-hydroxycotinine-O-β-D-glucuronide:



This is the first time that a metabolically formed nicotine phase 2 metabolite has been identified. Using MS and NMR spectrometry, this metabolite was found to be the O-β-D-glucuronide of THOC. In addition, evidence is given of further urinary nicotine phase 2 metabolites, *i.e.*, sulphuric acid conjugates of COT and THOC. The differences between our results and those reported in literature, but also between the published data itself concerning the quantitative results, can be explained by inter-individual variation in both nicotine phase 1 and phase 2 metabolism.

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Paper received: 10th September, 1992.